Lipid A controls the robustness of intratumoral accumulation of attenuated *Salmonella* in mice

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Engineered *Salmonella* have the potential to treat cancers that are not responsive to standard molecular therapies. This potential has not been realized because colonization in human tumors is insufficient and variable as shown in preliminary phase I trials. Recent studies have shown that *Salmonella* colonization is associated with an inflammatory response mediated by tumor necrosis factor (TNF). An injectable agent, molecular lipid A, could be used to control bacterial accumulation because it induces TNF production and is rapidly cleared. We hypothesized that concurrently administrating lipid A with attenuated *Salmonella* would increase intratumoral accumulation, improve the robustness of tumor-targeting and be nontoxic. To test this hypothesis, *Salmonella* and lipid A were injected into mice with 4T1 mammary tumors. Colonization was quantified after 48 hr using anti-*Salmonella* immunofluorescence. A 2 μg/mouse dose of lipid A increased the area of colonized tissue fourfold, reduced variance 50% and ensured colonization in all mice. Comparatively, *Salmonella* failed to colonize some control mice, similar to human trials. No toxicity was observed in any treated mice. The fraction of tumor tissue with more than 25% bacterial coverage was eight times greater for treated mice compared to controls. Lipid A treatment also reduced the maximum average distance of tissue to *Salmonella* colonies from 1348 to 260 μm. A mathematical model of bacterial drug production predicted that 2 μg lipid A would increase tumor cell death by 82%. These results suggest that lipid A could solve the clinical challenges of *Salmonella* therapy and enable safe and robust treatment of cancer with bacteria.

*Salmonella* have extraordinary potential as anticancer therapeutics\(^1\),\(^2\) that has not been realized because of poor colonization in human tumors.\(^3\)–\(^5\) Controlling inflammation may be the solution to this clinical challenge. When systemically injected into mice, *Salmonella* accumulate in tumors at densities 1000 times greater than normal tissue.\(^6\)–\(^8\) After being engineered to produce anticancer agents, *Salmonella* have been shown to inhibit tumor growth,\(^9\)–\(^12\) improve survival in mice\(^6\),\(^11\) and target regions that cannot be reached by molecular therapies.\(^14\)–\(^18\) In human trials, however, *Salmonella* have not been as effective.\(^3\)–\(^5\) In a phase I trial conducted with metastatic melanoma patients, colonization was low and there was considerable variability.\(^3\) Intratumoral accumulation was only observed in 17% of patients.\(^3\) In one patient, colonization was not detected by fine-needle aspiration but was identified when the lesion was excised,\(^3\) indicating that the bacterial distribution was spatially heterogeneous.\(^19\) To improve clinical efficacy, it will be necessary to increase accumulation density and reduce interpatient variability.

Several recent studies have suggested that inflammation plays a critical role in bacterial accumulation in tumors.\(^20\),\(^21\) In mice, intravenous injection of *Salmonella* (SL7207) increases the systemic concentration of tumor necrosis factor (TNF) and induces intratumoral hemorrhage.\(^20\) The TNF inflammation response increases blood influx and flushes bacteria into tumors.\(^20\) TNF induction also plays a central role in *Salmonella* toxicity because it is the primary cause of septic shock.\(^22\),\(^23\) The *Salmonella* strain used in all cancer clinical trials to date (VNP20009) has an msbB gene deletion that prevents proper formation of lipid A\(^24\) and reduces TNF induction by as much as 10,000-fold.\(^25\),\(^26\) Because of this deletion, VNP20009 does not cause sepsis and is safe in mice,\(^26\)–\(^28\) dogs,\(^29\) pigs\(^28\),\(^30\) and monkeys.\(^27\) Comparatively, VNP20009 does not accumulate in tumors as effectively as *Salmonella* with functional msbB.\(^16\),\(^21\) After intravenous administration, the tumor concentration of VNP20009\(^16\) is 1000 times lower than *Salmonella* strain SL1344 ΔaroA.\(^21\) The bacterial coverage in tumors from mice treated with wild-type *Salmonella* (SL1344) is dense and well-dispersed,\(^21\) whereas, in mice treated with VNP20009, the coverage is sparse and uneven.\(^7\),\(^16\) In VNP20009-treated mice, there is also considerable variation between individuals, even when mice and bacteria are genetically identical.\(^16\) These observations suggest that the msbB deletion, while essential for...
What’s new?
The bacteria *Salmonella*, infamous for causing disease, could be harnessed to treat cancer. Unfortunately, though they have been engineered to kill tumor cells, these bacteria fail to accumulate in human tumors. In this report, the authors propose that the genetic alteration necessary to make the bacteria safe also interferes with tumor-targeting ability. They tried administering the therapeutic bacteria together with the compound lipid A, which the engineered *Salmonella* can no longer produce. The addition of lipid A enhanced tumor targeting without restoring the bacteria’s toxicity, suggesting it could make *Salmonella* safe and effective for treating cancer.

Methods

Culture conditions and tumor formation

VNP20009 (*msbB*<sup>−</sup>, *purG*<sup>−</sup>, *sylF*<sup>−</sup>), previously derived from *Salmonella enterica* serovar Typhimurium ATCC 14028, was donated by Vion Pharmaceuticals (New Haven, CT). Bacteria were grown in LB broth or on LB agar at 37°C. 4T1 mammary carcinoma cells (American Tissue Type Collection, Manassas, VA) were grown in RPMI-1640 with 10% fetal bovine serum in a 37°C, 5% CO<sub>2</sub> incubator. Tumors were identified by immunofluorescence. Five micrometer thick equatorial sections were cut from excised tumors in the sagittal plane, deparaffinized, rehydrated and blocked with 1% bovine serum albumin for 20 min. A 1:200 dilution of polyclonal rabbit anti-*Salmonella* antibody (Abcam, Cambridge, MA) was applied at room temperature for 1 hr, followed by incubation with 1:250 polyclonal goat anti-rabbit Alexa 546 (Invitrogen, Carlsbad, CA) at room temperature for 1 hr. Processed tissue sections were counterstained with 0.1 μg/ml 4’,6-diamidino-2-phenylindole (DAPI; Thermo Scientific, Waltham, MA), mounted with coverslips, stored at 4°C and protected from light. Adjacent tumor sections were stained with hematoxylin and eosin (H&E) to identify tissue necrosis.

Safety, is the major cause of VNP20009’s increased variability and reduced tumor-targeting efficacy.

The *msbB* deletion alters the formation of lipid A by preventing a terminal acylation step that adds a myristic acid moiety. In its native form, lipid A from *Salmonella* is a potent stimulant of the immune system. When introduced into the blood stream, lipid A binds to toll-like receptor 4 (TLR4) on the surface of macrophages. TLR4 activates a signal transduction cascade that induces secretion of inflammatory cytokines, including TNF. Compared to live bacteria, soluble lipid A is more rapidly cleared from blood. Lipopolysaccharide from *Salmonella minnesota* R595, of which lipid A is the lipid component, has a plasma half-life of less than 5 min when injected intravenously in mice. Here, we show that controlling inflammation with molecular lipid A is the key to regulating *Salmonella* accumulation in tumors. We hypothesized that administering lipid A concurrently with attenuated *Salmonella* (*i*) increases intratumoral accumulation, (*ii*) improves the robustness of tumor-targeting and (*iii*) is nontoxic. To test these hypotheses, we injected escalating doses of lipid A and *Salmonella* into tumor-bearing mice. Immunofluorescence was used to quantify bacterial colonization, intermouse variability, local bacterial density and the contact distance between tumor cells and bacteria. Hematoxylin and eosin (H&E) staining was used to quantify the relationship between tumor necrosis and bacterial colonization. A mathematical model of bacterial drug production was created to predict the effect of lipid A on the synthesis of a protein drug, its diffusion out of bacterial colonies and the resultant death of surrounding cancer cells. A safe and robust method to control tumor colonization will enable the clinical use of bacterial therapy for a broad population of cancer patients.

Administration of *Salmonella* and lipid A

Diphosphoryl lipid A from *Salmonella minnesota* Re 595 (Sigma-Aldrich, St. Louis, MO) was dissolved in dimethyl sulfoxide at a concentration of 1 mg/ml. A dose of 2 × 10<sup>6</sup> colony forming units (CFU) mid-log phase VNP20009 was mixed with one of the three lipid A doses (0.1, 0.5 and 2 μg) in 100 μl PBS. These mixtures were injected intravenously into mice with size-matched tumors via the tail vein. Control mice were treated with VNP20009 in PBS without lipid A. Mice were sacrificed 48 hr after infection for tissue collection. Tumor and liver samples were fixed in 10% formalin and embedded in paraffin.

A second set of mice was used to measure bacterial density in livers. VNP20009, at a dose of 2 × 10<sup>6</sup> CFU/mouse, was injected into 20 mice with size-matched tumors, in combination with either saline (control) or 2 μg lipid A. After 48 hr, tumors and livers were removed, minced in saline and plated. LB agar plates were counted after growth at 37°C for 24 hr.

Immunofluorescence labeling

*Salmonella* were identified by immunofluorescence. Five micrometer thick equatorial sections were cut from excised tumors in the sagittal plane, deparaffinized, rehydrated and blocked with 1% bovine serum albumin for 20 min. A 1:200 dilution of polyclonal rabbit anti-*Salmonella* antibody (Abcam, Cambridge, MA) was applied at room temperature for 1 hr, followed by incubation with 1:250 polyclonal goat anti-rabbit Alexa 546 (Invitrogen, Carlsbad, CA) at room temperature for 1 hr. Processed tissue sections were counterstained with 0.1 μg/ml 4’,6-diamidino-2-phenylindole (DAPI; Thermo Scientific, Waltham, MA), mounted with coverslips, stored at 4°C and protected from light. Adjacent tumor sections were stained with hematoxylin and eosin (H&E) to identify tissue necrosis.

Lipid A controls *Salmonella* tumor-targeting

The addition of lipid A enhanced tumor targeting without restoring the bacteria’s toxicity, suggesting it could make *Salmonella* safe and effective for treating cancer.
Image acquisition

Images were acquired with an ORCA-HR monochrome CCD camera (Hamamatsu, Bridgewater, NJ) attached to an IX71 inverted epifluorescence microscope (Olympus, Center Valley, PA) with a Plan-APO 10× objective. *Salmonella*, labeled with Alexa 546, were identified using a green light excitation filter set D546/10×-565DCXT-E590LPv2 (Chroma, Rockingham, VT). DAPI counterstaining was identified with a UV excitation filter set AT350/50×-400DCLP-E420LPv2 (Chroma). H&E sections were acquired using a CRI MicroColor trichromatic filter (BioVision Technologies, Hopkinton, MA). A motorized xy-stage (Ludl Electronic Products, Hawthorne, NY) was used to create large (1 × 1 cm²) tiled images. To generate composite whole-section images, 250–300 adjacent 867 × 661 μm frames were acquired, scaled down 75% and tiled together.

Bacterial accumulation and distribution analysis

*Salmonella* accumulation was quantified using imageJ (National Institutes of Health, Bethesda, MD). Red-fluorescence, whole-tissue images were thresholded and converted into binary images to identify regions (pixels) that contained *Salmonella*. Artifacts were manually eliminated based on morphology and fluorescence intensity, using the unscaled high-resolution images that were acquired prior to tiling. The extent of *Salmonella* colonization was quantified by measuring the ratio of colonized tissue area (pixels with *Salmonella*) to total tissue area (total pixels). Equatorial tumor sections were assumed to be representative of whole tumors. The minimum detectable area fraction for this technique is 6.2 × 10⁻⁸. Local bacterial density was calculated by counting the number of *Salmonella*-containing pixels within a 100 μm-radius circle around each pixel in the whole-tissue sections. At a pixel resolution of 2.58 × 2.58 μm², a circular area of 100 μm radius contained 4792 pixels. Complete coverage (100%) indicated *Salmonella* in all 4792 pixels. Colonies were defined as regions with local densities greater than 25%. To determine tumor-to-*Salmonella* distances, a Euclidean distance map was generated around the bacterial colonies in every tumor section. This process assigned a value to each pixel indicating the distance of tumor cells at that location to the closest bacterial colony. An overall distance was determined by averaging all pixel values inside each tumor boundary. This value indicates the average distance from the ensemble of all tumor cells in a tumor to their closest bacterial colony. Tissue-average distances were calculated for each mouse in which bacterial accumulation was observed.

Histology

Tumor necrosis was manually identified using H&E-stained tumor sections. H&E images were reoriented to match the tissue contours in the adjacent immunofluorescence images that were used for *Salmonella* detection. Regions without nuclei or with disintegrated nuclei were classified as necrotic. Remaining regions with intact nuclei were classified as viable. The area of necrosis was calculated as the percentage of the total tissue areas. Maps of viable and necrotic regions were overlaid on immunofluorescence images to determine *Salmonella* colocalization. When establishing the correlation between areas of necrosis and *Salmonella* colonization, one tumor from the lipid-A group with a small necrosis ratio (44%) was excluded as an outlier.

Statistical analysis

One set of mice was used for all analyses, except liver colony counts. Pairwise comparisons between control and lipid A mice were conducted using the two-tailed Student’s *t*-test. The dose dependency of lipid A on bacterial colonization was tested using a one-way ANOVA model run in Matlab (MathWorks, Natick, MA). Coefficients of variance (CV) were calculated as the standard deviation normalized by the mean and were used to describe the variation of a parameter within a group of mice. Differences in variance between two groups of mice were determined using pairwise *F*-tests. This comparison of variances was used on several parameters, including tumor-to-*Salmonella* distance and area of necrosis. Pearson correlation coefficients were calculated to describe the correlations between necrotic area and the area of *Salmonella* colonization. In all the tests, statistical significance was determined at a *p*-value of 0.05.

Simulation of drug production and cancer cell death

A mathematical model was created to predict the effect of lipid A administration on bacterial drug delivery. The model was designed to use experimentally determined maps of *Salmonella* colonization to calculate (i) concentration profiles of a hypothetical protein drug and (ii) resultant patterns of tumor cell death.

\[
\frac{\partial C}{\partial t} = D \left( \frac{\partial^2 C}{\partial x^2} + \frac{\partial^2 C}{\partial y^2} \right) + BQe^{kt} - k_{deg} C \tag{1}
\]

\[
\frac{dN}{dt} = - \left( \frac{\mu_{max} C}{K_0 + C} \right) \cdot N \tag{2}
\]

The protein-drug balance (Eq. (1)) accounts for drug (C) diffusion in tumor tissue, bacterial drug production and drug degradation. Values for the drug diffusion coefficient, *D*, and the degradation rate, *k*<sub>deg</sub> (Table 1), were based on experimental values for biological macromolecules.\(^{34}\) Binary colonization matrices, *B*, described the intratumoral location of *Salmonella* and were derived from experimental immunofluorescence images. The drug production rate, *Q*, was derived from a measured protein expression capacity of 3500 molecules bacterium⁻¹·min⁻¹ for *Salmonella* in the intratumoral location of *Salmonella* and were derived from experimental immunofluorescence images. The drug production rate, *Q*, was derived from a measured protein expression capacity of 3500 molecules bacterium⁻¹·min⁻¹.\(^{35}\) Assuming that each *Salmonella* contained 2.6 × 2.6 × 5.0 μm³ in immunofluorescence images contained, on average, one bacterium. The intratumoral bacterial growth rate, *K*, was previously measured in our laboratory with VNP20009 in BALB/c mice.\(^{16}\) The cell viability (N) expression (Eq. (2)) describes the dynamics of drug-induced...
tumor cell death. The maximum tumor cell death rate, $\mu_{l \max}$, and the death rate saturation constant, $K_D$, were based on the parameters for chemotherapeutic drugs previously calculated in our laboratory.\textsuperscript{26}

Nondimensionalized equations were discretized using finite differences and solved using Matlab. In the simulations, drug production was induced 48 hr after $Salmonella$ injection (designated $t = 0$) and were run for 4 hr. At the initial time ($t = 0$), the drug concentration was zero ($C = 0$) and tumor cells were viable ($N = 1$) over entire tumors. A no-flux boundary condition for the diffusing drug was imposed at the tumor edge. Tumor cells were considered dead when $N$ dropped below 0.1. An independent simulation was run for each mouse. Statistical significance was determined by comparing predicted results from different groups of mice.

### Results

#### Lipid A increased $Salmonella$ accumulation

Injecting $Salmonella$ concurrently with lipid A lead to stronger and more robust intratumoral accumulation (Fig. 1). Lipid A-treated animals had more colonized area than controls 48 hr after injection (Fig. 1a). Accumulation increased with lipid A dose (Fig. 1b). A dose of 2 $\mu$g/mouse significantly increased colonized area nearly fourfold, from 0.38% in control mice to 1.53% ($p < 0.005$). Assuming that each colonized pixel contained one bacterium, these values are equivalent to an increase from $1.1 \times 10^6$ to $4.6 \times 10^6$ CFU/g. The progressive increase in $Salmonella$ accumulation was significantly dependent on the lipid A dose ($p < 0.05$). Lipid A administration also reduced inter-mouse variability. The CV for colonized area increased from 0.38% in control mice to 0.95% ($p < 0.005$; Fig. 2b). Correspondingly, 2 $\mu$g of lipid A reduced the tumor area with sparse (0–1%) coverage from 89.9% to 77.6% ($p < 0.005$, Fig. 2b). Tumors from the 2 $\mu$g lipid A group contained three times more colonies than the control tumors ($p < 0.005$ Fig. 2c). Lipid A did not significantly affect average colony size (Fig. 2d).

#### Lipid A reduced variability in the distance from tumor cells to $Salmonella$ colonies

Lipid A administration ensured short tumor-to-$Salmonella$ distances by increasing dispersion throughout tumors (Fig. 3). The tumor-to-$Salmonella$ distance indicates how far a bacterially produced protein drug would have to diffuse from its source. There was considerable variability in the average tumor-to-$Salmonella$ distance among control mice (Fig. 3b). Of the six tumors that showed bacterial accumulation, three had large areas that were far from $Salmonella$ (Fig. 3a, left, dark tissue regions). The distribution was more uniform in animals treated with lipid A (Fig. 3a, right). A 2 $\mu$g/mouse dose of lipid A reduced the standard deviation of the average tumor-to-$Salmonella$ distance among mice by 88% compared to controls (from 491 to 57$\mu$m; $p < 5 \times 10^{-5}$; Fig. 3b). This corresponded to a drop in the CV from 93% to 33% (Fig. 3b). The 2 $\mu$g/mouse dose reduced the maximum average distance from 1348 to 260 $\mu$m (Fig. 3b). Corresponding to the increase in uniformity, lipid A increased the percentage of tissue within 100 $\mu$m of $Salmonella$ from 30% to 55% ($p < 0.05$, Fig. 3c). This difference was predominantly due to significant increases in tissue within 10 $\mu$m of colonies ($p < 0.005$) and 10–20 $\mu$m from colonies ($p < 0.05$; Fig. 3d).

#### Lipid A increased colonization of viable tissue

Treatment with lipid A increased $Salmonella$ colonization in viable tissue and changed the relationship between accumulation and necrosis (Fig. 4). Bacterial localization within viable

### Table 1. Model parameters

<table>
<thead>
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<th>Symbol</th>
<th>Description</th>
<th>Value</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>$D$</td>
<td>Diffusion coefficient</td>
<td>7200 $\mu$m$^2$ h$^{-1}$</td>
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</tr>
<tr>
<td>$Q$</td>
<td>Drug production rate</td>
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<td>$K$</td>
<td>Bacterial growth rate$^1$</td>
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<td>$k_{avg}$</td>
<td>Drug degradation rate constant$^2$</td>
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<td>34</td>
</tr>
<tr>
<td>$\mu_{l \max}$</td>
<td>Maximum tumor cell death rate</td>
<td>2 h$^{-1}$</td>
<td>36</td>
</tr>
<tr>
<td>$K_D$</td>
<td>Death rate saturation constant</td>
<td>50 nM</td>
<td>36</td>
</tr>
</tbody>
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$^1$Equivalent to a doubling time of 16.8 hr.

$^2$Equivalent to a 15-hr half-life.
and necrotic tissue was determined by aligning H&E images (Figs. 4a and 4b) with images from adjacent sections used to identify Salmonella (using the same procedure as for the sections shown in Fig. 1). A 2 μg/mouse dose of lipid A increased colonization in viable tissue from 0.09% to 0.39% (p < 0.005, Fig. 4c). Lipid A also increased colonization of necrotic tissue (p < 0.005, Fig. 4e). Colonization ratios were higher in necrotic tissue compared to viable tissue for both lipid A (p < 0.005) and control (p < 0.05) mice (Fig. 4c).

In control mice, there was considerable variation in the extent of necrosis (CV = 0.50). The area of necrosis ranged from 6.7% to 89.6% (Fig. 4d). In comparison, the extent of necrosis was consistently high in lipid-A-treated mice (Fig. 4d). All but one of the lipid-A-treated tumors had between 60% and 80% necrosis. The distribution among lipid-A-treated mice (CV = 0.16) was significantly less compared to controls (p < 0.05, Fig. 4d). The correlation between Salmonella accumulation and the area of necrosis was different for the two groups of mice (Fig. 4e). For control mice, accumulation gradually increased (p < 0.05) with increasing necrosis (r = 0.84, Fig. 4e). For example, the tumor with the least necrosis (6.7%) showed no detectable bacterial accumulation. This relationship was steeper for lipid-A-treated mice (Fig. 4e). Injection of lipid A lessened the correlation between necrosis and bacterial accumulation (r = 0.61, p = 0.20). For...
these mice, the extent of necrosis was not as strong a predictor of colonization area.

**Mathematical prediction of drug production by *Salmonella***

A mathematical model was used to predict the efficacy of combined treatment with *Salmonella* and lipid A (Fig. 5). The model integrated three mechanisms of bacterial therapy: (i) protein–drug production by *Salmonella*, (ii) diffusion of the protein drug through tumor tissue and (iii) death of cancer cells in the presence of the protein drug. The simulations were initiated with two-dimensional tumor boundaries and *Salmonella* locations derived from immunofluorescence images (Fig. 1). Simulations were run for 4 hr and generated concentration profiles of a bacterially produced protein drug (Fig. 5a) and regions of induced cancer cell death (Fig. 5d). The average drug concentration in tumors from lipid-A-treated mice was predicted to be three times higher than controls ($p < 0.005$, Fig. 5b). This overall improvement was caused by an increase in the area of tissue that received high drug concentrations (1000–5000 nM, $p < 0.05$; >5000 nM, $p < 0.005$; Fig. 5c). The simulations predicted that lipid A would increase the area of predicted cell death by 82% ($p < 0.05$; Fig. 5e).
Lipid A increased the robustness of predicted drug delivery (Fig. 5c) and eliminated incidents of poor response (Figs. 5e and 5f). Mice treated with lipid A had significantly less variability in the area that did not receive the drug \((p < 0.0005; \text{Fig. 5c})\). The control group contained three mice in which more than 60% of the tumor did not contain any drug (Fig. 5c). The variability in predicted cell death was significantly higher \((p < 0.005)\) for the control group \((\text{CV} = 0.81)\) than the lipid A group \((\text{CV} = 0.13; \text{Figs. 5e and 5f})\). In four of the seven control mice, the area of induced cell death was less than 40% of the total area (Fig. 5f). Comparatively, in all seven lipid-A-treated mice, the predicted area of cell death was greater than 55% (Fig. 5f).

In addition to increasing predicted cell death in regions of high bacterial density, lipid A increased therapeutic efficacy in regions with low bacterial density (Fig. 5g) and far from colonies (Fig. 5h). The simulations predicted that tumor regions with local \textit{Salmonella} densities greater than 2% and local drug concentrations greater than 50 nM would be completely eliminated (Figs. 5a and 5d). In regions with local
densities less than 2%, lipid A reduced the predicted area of viable tissue from 61.8% to 30.2% ($p < 0.05$; Fig. 5g). Most predicted cell death occurred in regions with short tumor-to-\textit{Salmonella} distances (0–100 μm; $p < 0.05$) and was greater in the lipid-A-group ($p < 0.05$; Fig. 5h). Complementing this effect, lipid A also increased predicted cell death in tissue far from \textit{Salmonella} (200–300 μm, 300–400 μm, $p < 0.005$; 400–500 μm, $p < 0.05$; Fig. 5h).

**Discussion**

Administering lipid A concurrently with \textit{Salmonella} has the potential to overcome the limitations that prevent clinical use of bacterial therapy. We have shown that lipid A significantly increases bacterial accumulation and is nontoxic (Fig. 1). Lipid A also reduces variability and ensures colonization (Fig. 1c). Bacterial accumulation was seen in all 20 mice that received lipid A. In contrast, when VNP20009 was...
administered alone, some mice did not have bacterial colonization (Fig. 1c), similar to what was observed in clinical trials. Poor colonization was the most significant problem encountered in early human trials. This result confirms previous predictions that inflammation plays an essential role in bacterial colonization. The use of a separate, fast-clearing agent to induce inflammation in combination with noninflammatory bacteria will provide a safe method of increasing colonization and preventing variability.

Lipid A treatment also ensured uniform distribution of Salmonella colonies within tumors (Figs. 2 and 3). A more homogenous dispersion would increase the overall efficacy of

Figure 5. Mathematical simulation of protein drug production by Salmonella and prediction of tumor cell death induced by the drug. (a) Predicted local concentrations of a bacterially produced protein drug, 6 hr after induction. Scale bars are 2 mm. (b) Tumors from the lipid-A group (n = 7) were predicted to have higher (†, p < 0.005) average drug concentrations than controls (n = 7). (c) Mice treated with lipid A were predicted to have more tumor area (by percent of total) with produced drug concentrations between 1000 and 5000 nM (*, p < 0.05) and greater than 5000 nM (‡, p < 0.005). Error bars indicate standard errors of the mean. (d) Predicted area of dead tumor cells killed by the protein drug. Scale bars are 2 mm. (e) The model predicted that tumors from the lipid-A group would have a higher average area of tumor cell death than controls (*, p < 0.05). (f) The area of tumor cell death was predicted to be consistently high for lipid-A treated mice but more variable for control, untreated mice. (g) Lipid A reduced the predicted area of living tissue and increased the area of dead tissue in regions with sparse (less than 2%) bacterial coverage (*, p < 0.05). (h) Treatment with lipid A increased the predicted area of cell death in regions close (0–100 μm) and far (200–300, 300–400 and 400–500 μm) from bacterial colonies (†, p < 0.005; *, p < 0.05). Error bars indicate standard errors of the mean. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
bacterial therapy and increase the robustness of delivery. Higher bacterial densities (Fig. 2) and shorter tumor-to-Salmonella distances (Fig. 3) would make more of the tumor accessible to a bacterialy produced protein drug (Figs. 5a–5c). Mathematical modeling predicted that lipid A would significantly increase the area of affected tissue (Figs. 5d–5f). All tissue with more than 2% bacteria and most tissue closer than 100 µm to colonies would be completely eliminated (Figs. 5g and 5h). The model also predicted that lipid A treatment would have a self-amplifying effect by increasing tumor cell death in regions with low bacterial density and far from colonies (Figs. 5g and 5h). These cumulative benefits were achieved through elevated bacterial levels in surrounding tissue producing a diffusible protein drug.

The increased robustness in colonization observed after lipid A treatment was caused, in part, by a change in the relationship between bacteria and tumor necrosis. In the absence of lipid A, the extent of necrosis was a strong predictor of bacterial colonization (Fig. 4e). Salmonella are known to be attracted to molecules released by dying cells16–18 and to preferentially grow in necrotic tissue.27,37 Dependence on necrosis would explain why colonization was so heterogeneous in previous animal experiments8,16 and human trials.2,37 The extent of necrosis is highly variable in most tumors7 because it is caused by chaotic physiological conditions, for example, hypoxia and nutrient deprivation, that are shaped by disordered vasculature.38,39 Lipid A decoupled colonization from necrosis (Fig. 4e) by enhancing an alternate mechanism of bacterial accumulation. As shown by previous experimentation, lipid A increases systemic TNF levels,25 which in turn induces inflammation, increases blood influx and flushes bacteria into tumors.20 The independence of this mechanism was observed as the same proportional increase in bacterial density in both necrotic and viable tissue (Fig. 4c). From a therapeutic perspective, consistent targeting of viable tissue would be a considerable advantage because living tumor tissue is the primary target and delivery to necrotic tissue is unnecessary.

Administering attenuated Salmonella and lipid A separately would have several advantages. Using separate agents would enable (i) independent dose optimization and (ii) control over the timing of inflammation. The ability to independently tune bacteria and inflammation will be essential in clinical trials to account for the different immunological responses of mice and humans to Salmonella.20 As shown here, an inflammation response is an essential component of Salmonella’s tumor targeting and colonization. Because excessive inflammation would be toxic, a trade-off is necessary between manageable systemic inflammation and optimal therapeutic efficacy. The bacterial dose could be optimized to maximize tumor selectivity, and the lipid A dose could be independently optimized to induce a sufficient, but transient inflammatory response. Decoupling the timing of inflammation from bacterial injection would also enhance accumulation. In both mice and humans, serum TNF levels peak 1.5–2 hr after an intravenous challenge with lipopolysaccharide.31,32,41 Similarly, blood influx into tumors begins 2 hr after Salmonella injection and peaks 4–10 hr later.20 On the other hand, the number of Salmonella in the blood declines immediately after injection and, with a half-life of less than 30 min, the bacteria are almost completely cleared in 6 hr.27 Injecting lipid A ahead of Salmonella would induce TNF expression and increase blood influx at the correct time to maximize colonization and minimize the number of bacteria lost to clearance.

**Conclusion**

With its ability to initiate potent and robust tumor-invasion, lipid A is a direct solution to the insufficient and highly variable targeting that was problematic in clinical studies. Deleting the msbB gene improved the safety of Salmonella but reduced its tumor-targeting efficacy. The results presented here show that lipid A is an integral component of Salmonella’s tumor-targeting ability. Separately administering attenuated Salmonella and lipid A has the potential to attain the accumulation characteristics of wild-type Salmonella while maintaining clinical safety. The addition of lipid A into Salmonella-based cancer therapy may prove instrumental in realizing the promises of bacteria as tumor-targeting agents.

**References**